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Estrogenicity of 4-nonylphenol and diethylstilbestrol on in vitro oocyte maturation of the dusky tripletooth goby, *Tridentiger obscurus*

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This study investigated the estrogenicity of 4-nonylphenol (NP) and diethylstilbestrol (DES) in vitro during oocyte maturation in the marine fish, *Tridentiger obscurus*, using steroid hormone assays and GVBD assay. Vitellogenic (0.53 mm diameter) and fully vitellogenic (0.75 mm diameter) oocytes were in vitro exposed to NP (0.045–453.82 nM) and DES (0.037–372.62 nM). In vitellogenic oocytes, 45.38 and 453.82 nM NP and 3.73–372.62 nM DES increased the estradiol-17β (E2)/testosterone (T) ratio. In fully vitellogenic oocytes, 0.45, 45.38 and 453.82 nM NP and 3.73 nM DES increased E2/T. In the GVBD assay, 0.45 and 4.54 nM NP and 0.037, 3.73 and 37.26 nM DES inhibited GVBD. These results suggest that NP and DES have estrogen-agonistic effects in oocyte maturation in *T. obscurus*. In addition, NP and DES have different sensitivity according to the oocyte developmental stage, and the estrogen-agonistic effects of DES were greater than were those of NP.

Keywords: diethylstilbestrol; dusky tripletooth goby; estrogenicity; 4-nonylphenol; oocyte maturation

Introduction

4-Nonylphenol (NP) is a degradation product of nonylphenol ethoxylates (NPE), which are the major non-ionic surfactants used as plastics, pesticides, and industrial detergents (Talmage 1994; Maguire 1999; Servos. 1999; Ying et al. 2002). Many studies have reported that NP has weak estrogenic potency in fish. NP elevated the plasma concentrations of estradiol-17β (E2), vitellogenin, and zona radiata protein in both male and female fish and caused gonad abnormalities, such as the induction of ovotestes in males (Jobling et al. 1996; Gray and Metcalfe 1997; Arukwe et al. 1998; Ashfield et al. 1998; Kinnberg et al. 2000). These studies reported that NP acts as an estrogenic agonist and binds to the estrogen receptor (ER) and then elicits endocrine modulating effects affecting the reproduction of fish. Diethylstilbestrol (DES) is a synthetic estrogen. It was prescribed to women to inhibit abortion and as an estrogen supplement before its carcinogenic effects were recognized (Colborn et al. 1996: Kilp et al. 2002). In fish, exposure to DES increases vitellogenin production and decreases egg production (Folmar et al. 2000; Zhong et al. 2005).

Most studies of NP and DES have focused on their estrogenic potency, primarily using vitellogenin assays in males and females of freshwater fish species. Very few studies have examined the estrogenic potency of NP and DES on the maturation of marine species in vitro, although vitellogenesis and final oocyte maturation are consecutive process in reproduction.

During vitellogenesis, E2 is a key steroid, and final oocyte maturation is induced by progestins, which are involved in germinal vesicle break down (GVBD) in fish (Das and Thomas 1999; Patiño and Sullivan 2002). In addition, the inhibition of progestin-induced GVBD by kepone and 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane (*o*, *p*'-DDD) has been demonstrated in oocytes of the Atlantic croaker, *Micropogonias undulates* (Ghosh and Thomas 1995). However, recent studies reported DES-induced oocyte maturation in vitro, and the authors suggested that DES can bind to progestin receptors, mimicking progestin (Tokumoto et al. 2004; Baek et al. 2007).

The dusky tripletooth goby, *Tridentiger obscurus*, is among the most abundant fish species in estuaries of Cheju, Korea, and occurs in inshore waters along the coast and tidal pools of East China, Japan and Korea (Kim et al. 2005). This species is a good model organism in that they are small in size and reach maturity within a single year (Kaneko and Hanyu 1985). It has a multiple spawning pattern during spring and summer, i.e. from April to June, in Korea and the most of females die and males guard eggs after spawning (Jin et al. 2006).

The objectives of this study were to (1) investigate the estrogen-agonistic effects of NP and DES on oocyte

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maturation in vitro, and (2) compare the estrogenic potency using the GVBD assay and steroid hormone production in the dusky tripletooth goby. For steroid production, the ratio of E2 to testosterone (T) (E2/T ratio) was calculated as an index of endocrine disruption. The E2/T ratio has been cited as a sensitive biomarker of sex-steroid concentrations (Bevans et al. 1996; Folmar et al. 1996).

Materials and methods

Chemicals

4-Nonylphenol (Aldrich Chemical, Milwaukee, WI, USA) and DES (Sigma-Aldrich Chemical, St. Louis, MO, USA) were prepared as stock solutions (ng/mL) by dilution in ethanol. These were diluted further in ethanol. The ethanol concentration in the incubation medium was kept at less than 0.1%. Standard testosterone (T) and estradiol-17β (E2) were purchased from Sigma Chemical (USA). Antiserum for T was purchased from Sigma Chemical (USA), and that for E2 was a kind gift from Dr. Alexis Fostier (INRA, France). Radioactive [2,4,6,7-^{3}H]testosterone and [2,4,6,7-^{3}H] 17β-estradiol were obtained from Amersham Life Science (England).

Experimental fish and oocyte incubation in vitro

Four *T. obscurus* were captured by a scoop net (4×4 mm in mesh size) in tidal pools at Pyoseon, Jeju, Korea, during the spawning season (April–June) and used for this study. Fishes were anesthesized with 2-phenoxyethanol (0.3 mL/L) and dissected. After dissection, the ovaries were removed and placed in ice-cold balanced salt solution (132.96 mM NaCl, 3.09 mM KCl, 0.28 mM $MgSO_4 \cdot 7H_2O$, 0.98 mM $MgCl_2 \cdot 6H_2O$, 3.40 mM $CaCl_2 \cdot 6H_2O$, and 3.65 mM HEPES). The oocytes were isolated and gathered using fine forceps under the condition of ice-cold balanced salt solution. Oocytes with average diameters of 0.53 and 0.75 mm were used for incubation. Approximately 20 follicle-enclosed oocytes were incubated in each well of 24-well culture plates containing 1 mL of Leibovitz's L-15 medium (Gibco).

In this study, we performed two separate similar experiments; each experiment used oocytes from individual fish. In experiment I, oocytes measuring 0.53 mm in diameter were exposed to 4.54, 45.38 and 453.82 nM NP and 3.73, 37.26 and 372.62 nM DES. In experiment II, oocytes measuring 0.75 mm in diameter were exposed to 0.045, 0.45, 4.54, 45.38 and 453.82 nM NP and 0.037, 0.37, 3.73, 37.26 and 372.62 nM DES.

The plates were incubated for 24 h at 18°C with constant gentle shaking. The pH and osmolarity of the medium was adjusted to 7.62 and 290 mOsm, respectively. After incubation, we analyzed the steroid production from 0.53-mm-diameter oocytes and both the GVBD assay and steroid production from 0.75-mm-diameter oocytes. The incubation medium was collected and stored at −80°C until measurement of the sex steroid levels. The 0.75-mm-diameter oocytes were fixed with clearing solution (ethanol:formalin:glacial acetic acid, 6:3:1). The location of the germinal vesicle (GV = nucleus) was observed under low-power magnification using a dissecting microscope. The number of oocytes completing GVBD, i.e. dissolution of the nucleus, was counted in each well and calculated as a percentage.

Histological observation of oocytes

Some pieces of ovary from each individual were fixed in Bouin's solution for 24 h. The fixed samples were washed, dehydrated and embedded with paraffin. Serial sections of 4–6 μm thickness were prepared and slides were stained in Mayer's hematoxylin and 0.5% eosin and mounted with malinol. Histological samples were observed through light microscope (BX50, Olympus, Japan).

Radioimmunoassay (RIA)

After incubation, steroids in aliquots of medium were extracted twice using five volumes of ethylacetate: cyclohexane (1:1). Then, the T and E2 levels were measured by RIA following Kobayashi et al. (1987). The intra-assay coefficients of variance were 2.3% ($n = 3$) and 3.4% ($n = 3$) for the T and E2 assays, respectively, and the respective inter-assay coefficients of variance were 12.5% ($n = 5$) and 11.5% ($n = 5$). The minimum detectable limits were 10 and 12.5 pg/mL for T and E2, respectively.

Statistics

All data were expressed as means with the standard error of the means (SEM) and tested for normality using the Kolmogorov–Smirnov test using SPSS software (version 17.0) for Windows (SPSS, Chicago, IL, USA). A non-parametric Kruskal-Wallis test followed by the Bonferroni adjustment was used due to the assumptions of normality and equal variance were failed. A value of $P < 0.05$ was considered statistically significant.

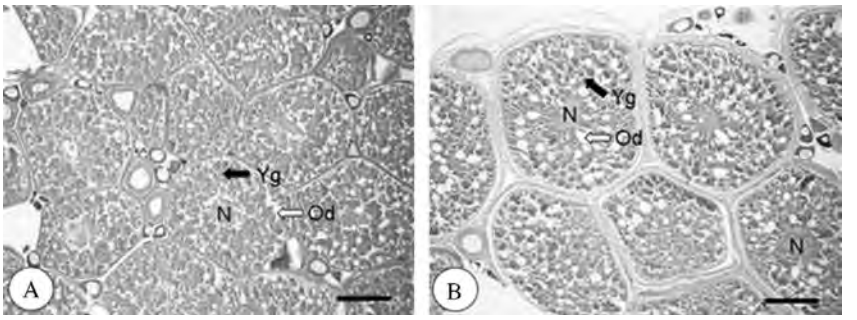

Figure 1. Histological observations of oocytes from dusky tripletooth goby. A, oocytes of 0.53 mm; B, oocytes of 0.75 mm. Scale bars indicate 200 μm. N, nucleus; Od, oil droplet; Yg, yolk granule.

Results

Histological observation of oocytes

In 0.53-mm-diameter oocytes, the yolk granules (Yg) were spread throughout the ooplasm and the oil droplets (Od) were distributed over the ooplasm (Figure 1A). In 0.75-mm-diameter oocytes, Yg continued to increase in number and fill the entire ooplasm (Figure 1B). Ods of 40–50 μm were observed around the nucleus and the nucleus was still in the middle of ooplasm.

Effects of NP and DES on sex-steroid hormone production

We analyzed steroid production by 0.53-mm-diameter oocytes and both the GVBD assay and steroid production using 0.75-mm-diameter oocytes after incubation. The 0.53-mm oocytes were the vitellogenic stage, and the 0.75-mm oocytes were the fully vitellogenic stage.

With the 0.53-mm oocytes (Figure 2), exposure to 45.38 nM NP resulted in a significant increase in the

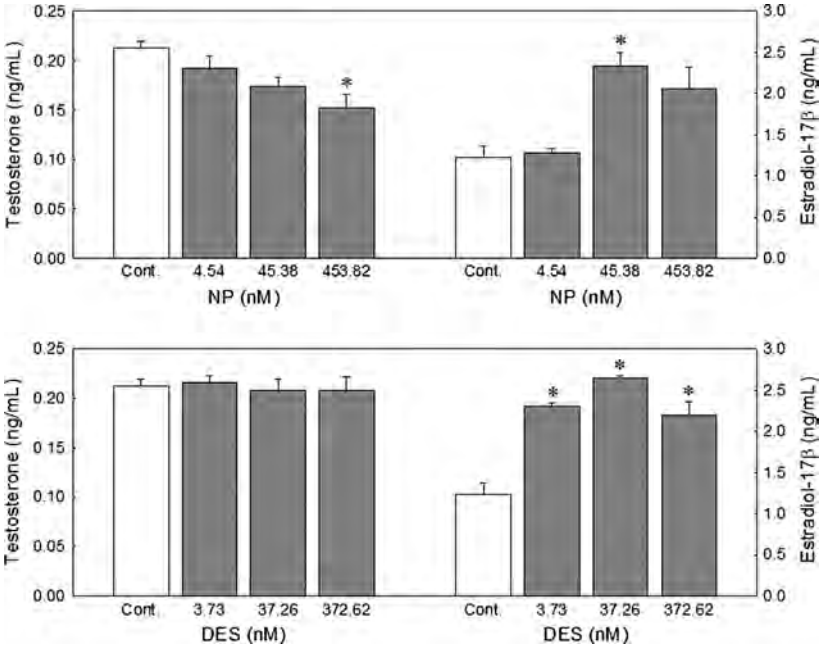

Figure 2. Effects of different NP and DES concentrations on in vitro testosterone and estradiol-17β production in dusky tripletooth goby oocytes (oocyte diameter = 0.53 mm) after a 24-h incubation. Values are means ± SEM of the concentrations of each steroid in three replicate wells with 20 oocytes/well. Controls were incubated with only incubation medium. Data were analyzed using the Kruskal-Wallis test followed by Bonferroni adjustment. Asterisks show significant differences from controls ($P < 0.05$).

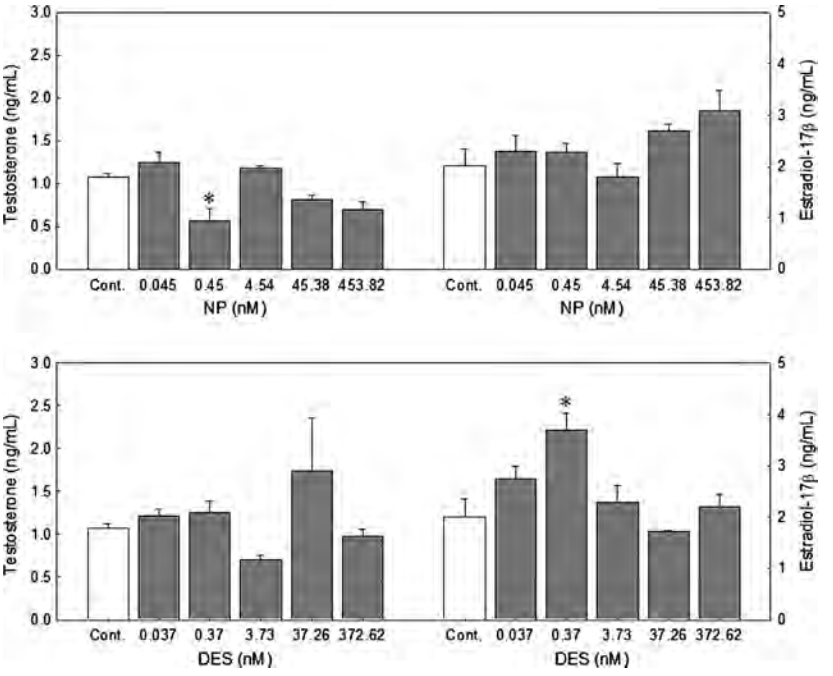

Figure 3. Effects of different NP and DES concentrations on *in vitro* testosterone and estradiol-17β production in dusky tripletooth goby oocytes (oocyte diameter = 0.75 mm) after a 24-h incubation. Values are means ± SEM of the concentrations of each steroid in three replicate wells with 20 oocytes/well. Controls were incubated with only incubation medium. Data were analyzed using the Kruskal-Wallis test followed by Bonferroni adjustment. Asterisks show significant differences from controls ($P < 0.05$).

production of E2 compared with controls (2.33 ± 0.24 vs. 1.23 ± 0.24 ng/mL), whereas 453.82 nM NP resulted in a significant decrease in production of T compared with controls (0.15 ± 0.02 vs. 0.21 ± 0.01 ng/mL). DES had no significant effects on T production, whereas all tested concentrations of DES resulted in significant increases in the production of E2 (2.31 ± 0.04, 2.65 ± 0.04 and 2.20 ± 0.23 ng/mL) compared with controls (1.23 ± 0.24 ng/mL) ($P < 0.05$).

For the 0.75-mm oocytes (Figure 3), exposure to 0.45 nM NP resulted in a significant decrease in the production of T compared with the controls (0.57 ± 0.25 vs. 1.07 ± 0.08 ng/mL), whereas no significant effect on E2 production was found. With DES treatment, 0.37 nM of DES resulted in a significant increase in E2 production (3.70 ± 0.45 ng/mL), but no significant effect on T production was observed.

Because the E2/T ratio has been cited as a sensitive biomarker of abnormal sex-steroid concentrations (Bevans et al. 1996; Folmar et al. 1996), we calculated the E2/T ratio as an index of endocrine disruption. In the 0.53-mm oocytes (Figure 4), 45.38 and 453.82 nM NP increased the E2/T (13.06 ± 2.52 and 12.69 ± 3.29) and every dose of DES increased E2/T (10.70 ± 0.93,

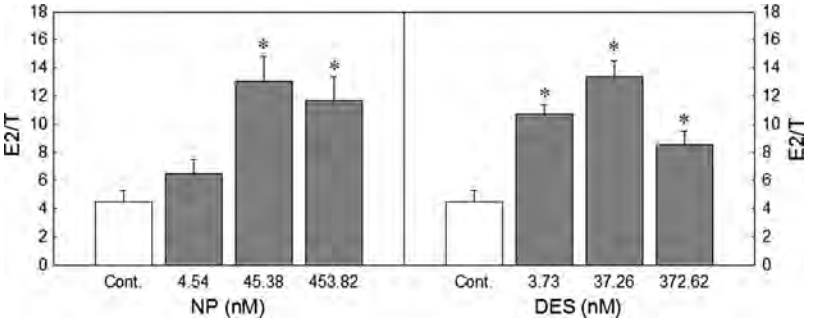

Figure 4. Effects of different NP and DES concentrations on E2/T ratio in dusky tripletooth goby oocytes (oocyte diameter = 0.53 mm) after a 24-h incubation. Values are means ± SEM of the ratio of each steroid in three replicate wells with 20 oocytes/well. Controls were incubated with only incubation medium. Data were analyzed using the Kruskal-Wallis test followed by Bonferroni adjustment. Asterisks show significant differences from controls ($P < 0.05$).

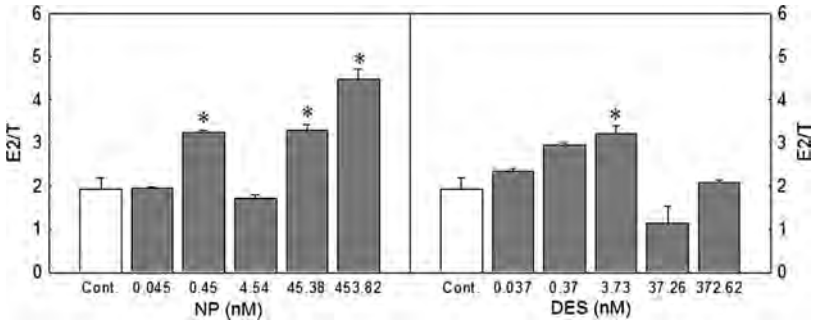

Figure 5. Effects of different NP and DES concentrations on E2/T ratio in dusky tripletooth goby oocytes (oocyte diameter = 0.75 mm) after a 24-h incubation. Values are means ± SEM of the ratio of each steroid in three replicate wells with 20 oocytes/well. Controls were incubated with only incubation medium. Data were analyzed using the Kruskal-Wallis test followed by Bonferroni adjustment. Asterisks show significant differences from controls ($P < 0.05$).

13.35 ± 1.65, and 8.52 ± 1.40) compared with controls ($P < 0.05$). In the 0.75-mm oocytes (Figure 5), 0.45, 45.38 and 453.82 nM NP increased E2/T (3.25 ± 0.08, 3.29 ± 0.25, and 4.49 ± 0.38); and 3.73 nM DES increased E2/T (3.23 ± 0.32) compared with controls ($P < 0.05$).

Effects of NP and DES on GVBD

We examined the effects of NP and DES on GVBD using 0.75-mm oocytes (Figure 6). Treatment with 0.45 and 4.54 nM NP resulted in a significant decrease in GVBD compared with controls (12.12 ± 2.62 vs. 4.65 ± 0.15, 12.12 ± 2.62 vs. 0, $P < 0.05$). None of the other treatments had significant effects on GVBD (i.e. 0.045, 45.38 and 453.82 nM NP). Treatments with 0.037, 3.73 and 37.26 nM DES resulted in a significant decrease in GVBD (5.63 ± 0.88 and 0) compared with controls ($P < 0.05$).

Discussion

Histological observation of gonad from *T. obscurus* indicated that 0.53 mm oocytes were vitellogenic stage and 0.75 mm oocytes were fully vitellogenic stage. This study investigated the estrogenicity of NP and DES on the maturation of *T. obscurus* oocytes in vitro by measuring steroid production and the GVBD assay using oocytes at different developmental stages. NP at 453.82 nM decreased T production, and 45.38 nM NP increased E2 production by the 0.53-mm oocytes (vitellogenic oocytes), whereas NP had no significant effects on E2 production by the 0.75-mm oocytes (fully vitellogenic oocytes). The E2/T ratio, used as an index of endocrine disruption, was increased by 45.38 and 453.82 nM NP in oocytes of both stages. Moreover, 0.45 and 4.54 nM NP inhibited GVBD. These results suggest that NP has estrogen-agonistic effects on the development and maturation of *T. obscurus* oocytes in vitro.

Recently, Baek et al. (2003) reported that NP stimulated in vitro estrogen synthesis in fully vitellogenic oocytes of the longchin goby, *Chasmichthys dolichognathus*. Hwang et al. (2008) also reported that NP has estrogenic potency in vitro on fully mature and vitellogenic oocytes by increasing E2/T and E2/17α20βOHP in the greenling, *Hexagrammos otakii*, a marine multiple-spawning species. In the fathead

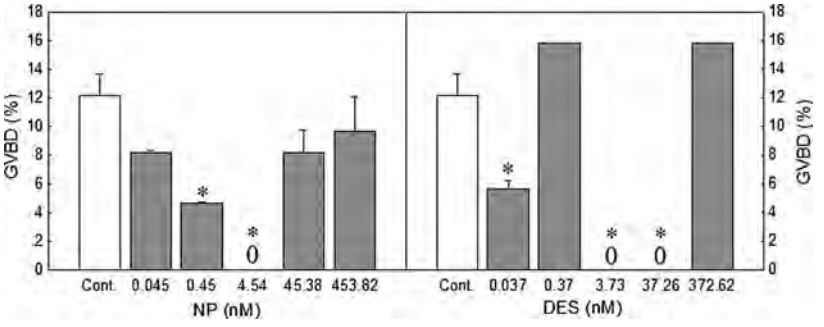

Figure 6. Effects of different NP and DES concentrations on in vitro GVBD in dusky tripletooth goby oocytes (oocyte diameter = 0.75 mm) after a 24 h incubation. Values are means ± SEM of the percentage of GVBD in three replicate wells with 20 oocytes/well. Controls were incubated with only incubation medium. Data were analyzed using the Kruskal-Wallis test followed by Bonferroni adjustment. Asterisks show significant differences from controls ($P < 0.05$).

minnow, *Pimephales promelas*, a freshwater multiple-spawning species, NP increased the plasma E2 levels and decreased egg production, although NP decreased plasma vitellogenin levels at the beginning of the breeding season (Giesy et al. 2000). The induction of vitellogenin is reported to be one of the most sensitive biomarkers for assessing estrogenic effects (Heppell et al. 1995; Sumpter and Jobling 1995). However, Giesy et al. (2000) reported that the vitellogenin assay is inappropriate for multiple-spawning species such as the fathead minnow because plasma E2 levels remain high during the spawning period. In addition, they concluded that NP increased endogenous E2 levels and then inhibited egg production via an indirect mechanism and not a direct estrogen-agonistic mechanism. In our study, NP increased endogenous E2 production at the vitellogenic stage and inhibited GVBD at the fully vitellogenic stage. In fact, the fully vitellogenic stage is the threshold stage in the maturation process, during which E2 levels decrease and progestin levels, such as 17α-hydroxy,20β-dihydroprogesterone (17α20βOHP) or 17α,20β,21-trihydroxy-4-pregnen-3-one (17α20β21P) increase (Rocha and Reis-Henrique 1998, 2000; Pankhurst and Riple 2000). In this regard, it is possible that the inhibition of GVBD is influenced by elevated E2 levels following NP exposure, although we have no data on progestin levels in the oocytes of *T. obscurus*.

The synthetic estrogen DES has been used as an estrogen supplement (Colborn et al. 1996; Kilp et al. 2002) and acts as a ligand for estrogen receptors (Prins et al. 2001). In addition, DES is reported to increase vitellogenin levels and its mRNA expression and to inhibit reproductive success in minnows (Folmar et al. 2000; Zhong et al. 2005). In our study, 3.73, 37.26 and 372.62 nM DES increased E2 production by vitellogenic oocytes, and 0.37 nM DES increased E2 production by fully vitellogenic oocytes, whereas no significant effects on T production were observed for oocytes at both stages. Moreover, DES increased the E2/T ratio of oocytes at both stages and inhibited GVBD. As Yang et al. (2008) noted, DES has an estrogenic effect by increasing E2 levels and decreasing T levels. In addition, DES induced feminization and vitellogenin production and inhibited the gonadosomatic index (GSI) and number of eggs spawned in the Chinese rare minnow, *Gobiocypris rarus* (Zhong et al. 2005). Our results, together with those of the previous studies, suggest that DES is an estrogen-agonist or progestin-antagonist in the maturation of *T. obscurus* oocytes.

However, DES inhibited estrogen synthesis but induced progestin synthesis and GVBD *in vitro* using mature oocytes of *C. dolichognathus* (Baek et al. 2003, 2007). Moreover, DES induced final maturation of goldfish and zebrafish oocytes in vitro, and it is suggested that DES has progestin-agonistic effects by binding ovarian membrane progestin receptor (Tokumoto et al. 2004, 2005, 2007); the progestin-agonistic activity of DES was approximately 1% that of 17α20βOHP. In these studies, DES has progestin-agonistic effects which are opposite to the other studies, in which DES has estrogen-agonistic effects, including our previous results. The reasons for these different effects of DES are unknown. However, taking together all of these studies on the effects of DES, DES may act as a ligand for the progestin receptor as well as for the estrogen receptor, varying from species to species, and the precise binding site of DES needs to be studied, as noted by Tokumoto et al. (2007).

Based on our results, NP and DES exhibited estrogen-agonistic effects on sex steroid hormone production and the oocyte maturation process, and they have different sensitivity according to the oocyte developmental stage of *T. obscurus*. In fact, the effects of certain chemicals are different not only from species to species but from individual to individual due to differences in physiological conditions. Moreover, the results would differ depending on exposure conditions, analytical systems and assay protocols. In this regard, our results indicated that lower concentrations of NP and DES inhibited GVBD significantly compared to controls. Moreover, there was no significant increase in GVBD by exposure to NP or DES. Also, different responses among exposure groups would result from different effective concentrations for a response to these chemicals.

In addition, the estrogen-agonistic effects of DES by inhibiting GVBD were greater than were those of NP. These results provide useful information about the maturation process in response to endocrine disruption using sexually mature female dusky tripletooth gobies.

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